bio-protocol Preprint

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Expression analysis

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An abbreviated version of this protocol was published in eLIFE in Jul 2015

Seasonal shift in timing of vernalization as an adaptation to extreme winter

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Detailed protocol

Expression Analysis

1) Start with **RNA Extraction** (Protocol adapted from Box et al., 2011. doi: https://doi.org/10.1186/1746-4811-7-7). Collect ~200mg of above soil tissue into 1.5mL micro-centrifuge tubes that each contain a 3mm tungsten-carbide bead. Freeze immediately by immersing the tubes in liquid nitrogen.

2) Cool GenoGrinder 96 wells cryo-adapter blocks by covering with dry ice for 15 mins.

3) Transfer the micro-centrifuge tubes into the pre-cooled blocks and grind the frozen tissue into a fine powder using 30 second 1200 rpm setting on a GenoGrinder 2010 bead mill (SpexSamplePrep, New Jersey, USA).

Warning! RNA Extraction buffer contains Phenol, Chloroform and 2-mercaptoethanol. Steps 4 - 8 must be carried out in a fume cupboard and suitable protective clothing and eyewear must be worn.

4) For 192 RNA extractions take 60 mL of stock RNA extraction buffer (0.1 M Tris pH8.0, 5 nM EDTA pH 8.0, 0.1 M NaCl, 0.5% SDS). Add 600 µL 2-mercaptoethanol and warm the solution to 60 ^{o}C.

5) Add 300 µL of warmed, extraction buffer to each sample and thoroughly mix by pipetting.

6) Ensure that the tissue is fully suspended before adding 300 µL 1:1 acidified phenol pH 4.3 ± 0.2: Chloroform.

7) Mix tubes vigorously for 10 minutes at RT and then spin down in a micro-centrifuge at RT for 15 minutes at maximum speed (for this protocol maximum speed centrifugation refers to a force > 4000 x g).

8) For each sample carefully remove the top layer of supernatant only and transfer to a new 1.5mL micro-centrifuge tube.

9) Add 240µL of isopropanol (2-propanol) and 30µL of 3M sodium acetate (pH 5.2) to each sample and leave nucleic acids to precipitate at -80^{o}C for 15 minutes.

10) Spin samples down for 30 minutes at maximum speed in a refrigerated centrifuge set at 4^{o}C.

11) Carefully remove the supernatant and avoid disturbing the pellet at the bottom of each tube.

12) Wash the pellets twice with 750 µL of 70% ethanol with five minute maximum speed centrifugation steps after each wash. Take care not to disrupt the pellet during these wash steps.

13) Carefully removed any residual ethanol using a fine pipette tip and leave pellets to air dry at room temperature for 15 minutes.

14) Re-suspend RNA pellets in 500µL RNase free water, add 500µL 4M LiCl to each sample then leave for genomic DNA digestion overnight at 4^{o}C.

15) Centrifuge samples at maximum speed for 30 minutes at 4^{o}C to pellet the RNA.

16) Pipette off and dispose of the supernatant containing genomic DNA.

17) Wash pellet twice with 750µL of 70% ethanol with five minute maximum speed centrifugation steps after each wash. Take care not to disrupt the pellet during these wash steps.

18) Carefully removed any residual ethanol using a fine pipette tip and leave pellets to air dry at room temperature for 15 minutes.

19) Suspend the RNA in 50 µL RNase free water.

20) Determine RNA concentration and quality for each sample using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Denver USA). RNA preparations with an A_{260}/A_{280} ratio of ~2.0 and A_{260}/A_{230} ratio >2.2 were considered suitable for use downstream without further purification.

21) Dilute each sample to 200 ng μL^{-1} using RNase free water.

22) Next carry out **Reverse Transcription.** For this publication, we used Nano-Script Reverse Transcription Kit reagents from Primerdesign (Southampton, UK) and their two step reaction protocol.

23) Set up Reaction 1 for each sample as shown below:

8 µL	RNase free water
1 µL	Oligo (d)T primer
5 µL (1 µg)	Total RNA (200 ng μL^{-1})

24) Incubate the samples for 5 minutes at 65 ^{o}C then transfer onto ice before addition of the following:

2 µL	10X buffer
1 µL	dNTP mix
2 µL	DTT
4 µL	PCR grade water
1 µL	Reverse transcription enzyme

25) Commence Reaction 2 as shown below:

55 ^{o}C	20 minutes
5 ^{o}C	15 minutes

26) Dilute cDNA samples by adding 180 µL of RNase free PCR grade water.

27) For **Quantitative Polymerase Chain Reaction (qPCR)** we used a LightCycler 480 II instrument (Roche Life Science, Germany) in conjunction with probes from the LightCycler 480 Universal Probes Library (UPL) range and gene specific primers to determine expression levels. Primers and Specific Universal Probes used for this publication are shown below:

***FLC* (AT5G10140)**	
sFLC_UPL_#65_F	5'-gtgggatcaaatgtcaaaaatg-3'
sFLC_UPL_#65_R	5'-ggagagggcagtctcaaggt-3'
UPL #65	5'-ctggagga-3'
***VIN3* (AT5G57380)**	
VIN3_UPL_#67_F	5'-cgcgtattgcggtaaagataa-3'
VIN3_UPL_#67_R	5'–tctctttcgccaccttcact-3'
UPL #67	5'-ctccagca-3'
***FT* (AT1G65480)**	
FT_UPL_#138_F	5'-ggtggagaagacctcaggaa-3'
FT_UPL_#138_R	5'-ggttgctaggacttggaacatc-3'
UPL #138	5'-tggtggat-3'
***UBC* (AT5G25760)**	
UBC_UPL_#9_F	5'-tcctcttaactgcgactcagg-3'
UBC_UPL_#9_R	5'-gcgaggcgtgtatacatttg-3'
UPL#9	5'-tggtgatg-3'

28) We used an EpiMotion robot (Eppendorf, Hamburg, Germany) to pipette triplicates of 6.5µL qPCR reactions into 384 well plates (Roche Life Science, Germany):

5 µL	Roche Universal Probes Library master mix
0.1 µL	200 mM Forward primer
0.1 µL	200 mM Reverse primer
0.4 µL	Universal Probe Library Probe

0.9 µL	PCR grade water

29) The following thermo-cycler program was used to amplify cDNA.

Step	Temperature	Time
1	95 °C	10 minutes
2	95 °C	10 seconds
3	60 °C	20 seconds
4	72 °C	1 second
45 cycles repeating steps 2-4		
Cooling	40 °C	10 seconds

30) To determine relative levels of gene expression we used the comparative Ct method (Schmittgen, T. D. & Livak, K. J. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc,* 3**,** 1101-8) and carried out statistical analysis using GraphPad Prism (La Jolla, California, USA).

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Duncan, S. (2020). Expression analysis. Bio-protocol Preprint. bio-protocol.org/prep383.
2. Duncan, S., Holm, S., Questa, J., Irwin, J., Grant, A. and Dean, C.(2015). Seasonal shift in timing of vernalization as an adaptation to extreme winter. eLIFE. DOI: 10.7554/eLife.06620